VITAMIN K EPOXIDASE: DEPENDENCE OF EPOXIDASE ACTIVITY ON SUBSTRATES OF THE VITAMIN K-DEPENDENT CARBOXYLATION REACTION

J. W. SUTTIE, L. O. GEWEKE, S. L. MARTIN+ and A. K. WILLINGHAM+

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, WI 53706 and *+Department of Biochemistry, Kirksville College of Osteopathic Medicine, Kirksville, MO 63501, USA*

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1. Introduction

Vitamin K catalyzes the post-translational carboxylation of specific glutamyl residues in precursor proteins to form γ-carboxyglutamyl residues which are present in prothrombin and other vitamin K-dependent proteins. These precursors increase in the livers of vitamin K-deficient rats, and the carboxylation of both endogenous protein precursors and low molecular weight peptide substrates can be demonstrated in detergent-solubilized microsomal preparations [1,2].

The same microsomal preparations which carry out this O_2-dependent carboxylation reaction will also catalyze the conversion of vitamin K to its 2,3-epoxide (epoxidase activity). Observations that the enzymatic formation of the vitamin K epoxide is elevated in livers of vitamin K-deficient rats, which also contain high concentrations of prothrombin precursors, led to the proposal that epoxidation of vitamin K is associated with the action of the viatamin in promoting prothrombin synthesis [3]. This hypothesis has been strengthened by studies of the effects of various anticoagulants in normal and Warfarin-resistant rats [4–7] by observations that many of the requirements for the epoxidation and carboxylation reactions are similar [8], by observations of the specificity and distribution of this activity [9,10], and by evidence of a common vitamin K intermediate required for both reactions [11]. These studies (reviewed [12]) support the concept that the formation of vitamin K epoxide is coupled to the vitamin K-dependent carboxylation event; but they are not conclusive.

This report presents additional evidence that epoxidation of vitamin K is associated with the vitamin K-dependent carboxylation reaction. A synthetic peptide (Phe–Leu–Glu–Glu–Leu), which serves as an exogenous substrate for the carboxylation reaction, has been shown to stimulate the vitamin K epoxidase activity; and immunospecific removal of prothrombin precursors from Triton-solubilized microsomes has been shown to lower the vitamin K epoxidase activity.

2. Methods

For the data in table 1, Triton X-100 solubilized rat liver microsomes were obtained from fasted male Sprague-Dawley rats (250–350 g) treated with Warfarin (5 mg/kg, i.p.) 18 h before they were killed by decapitation. The livers were quickly removed and chilled in homogenizing buffer (0.25 M sucrose, 25 mM imidazole (pH 7.2)) minced, homogenized in 3 vol. homogenizing buffer and centrifuged at 105 000 × *g* for 1 h. The microsomal pellet was surface washed with homogenizing buffer and subsequently homogenized in the same buffer which also contained 2% Triton X-100 and 0.2 M KCl to solubilize the microsomal membranes. These solubilized microsomes (=0.75 g liver/ml) were centrifuged at 105 000 × *g* for 45 min to remove any insoluble material. For the data in fig.1, a similar Triton-solubilized preparation was obtained from vitamin K-deficient Holtzman strain rats as in [13]. This procedure was modified by the inclusion of 1 mM dithiothreitol in all buffers. Details of the carboxylase and epoxidase incubation are indicated in the appropriate legends. The amount of vitamin K epoxide formed in the incubation was determined by extraction with isopropanol/hexane (3/2), and reverse phase thin-layer chromatography as in [4,8]. Determination of

vitamin K-dependent incorporation of $H^{14}CO_3^-$ into endogenous microsomal proteins [13] and exogenous peptide substrates [14,15] has been described. For immunospecific adsorption, solubilized microsomal preparations were incubated with anti-prothrombin/Sepharose (25 μl wet wt vol./ml microsomes) for 60 min at 24°C. Immunoadsorbent was removed by low-speed centrifugation and the supernatant assayed for epoxidase activity or $H^{14}CO_3^-$ incorporation. The immunoadsorbent used was prepared as in [16–18].

[5,6,7,8-^{3}H]Vitamin K_1 (7 Ci/mmol) was synthesized as in [19] and $NaH^{14}CO_3$ (60 mCi/mmol) and NCS (tissue solubilizer) were purchased from Amersham Searle (Arlington Heights, IL). NADH, Triton X-100, *Echis carinatus* venom, and dithiothreitol were purchased from Sigma Chem. Co. (St Louis, MO). Phe–Leu–Glu–Glu–Leu was obtained from Vega Chem. (Tucson, AZ). Sodium Warfarin was a gift from Endo Labs. (Garden City, NY), and all other chemicals were analytical reagent grades.

3. Results

Previous studies have demonstrated that vitamin K epoxidase activity is elevated in liver microsomal preparations from vitamin K-deficient rats. These preparations contain high levels of prothrombin precursors, and the available data do not indicate whether the stimulation of epoxidase activity is due to the presence of these precursors or to an induction of the epoxidase in the deficient state. Potential thrombin activity in solubilized microsomes can be removed by incubation in the presence of antiprothrombin/Sepharose [17], and the effect of specific removal of these precursor proteins on vitamin K epoxidase activity and vitamin K-dependent carboxylation was determined. The results (table 1) indicate that ~20–25% of the $^{14}CO_2$ which was fixed into microsomal proteins in the presence of vitamin K could be removed by treatment with anti-prothrombin/Sepharose. Treatment of the microsomes with this immunoadsorbent also decreased the epoxidase activity by ~35%. The data also reveal that, within the limits of the assay, this procedure was effective in removing all antigenically-active prothrombin species from the microsomes. The epoxidase activity in normal rats is only ~30 of that in Warfarin-treated rats , and treatment of solubilized microsomes from normal rats with anti-prothrombin/Sepharose also resulted in a decrease of ~30% in vitamin K epoxidase activity (data not shown).

These data demonstrated that removal of a major portion of the microsomal endogenous precursor

Table 1
Effect of immunospecific adsorption on microsomal vitamin K-dependent carboxylation, vitamin K epoxidase activity, and prothrombin precursor activity

	Precursor conc. (U/g liver)		^{14}C-Labeled protein (dpm/g liver × 10^{-3})		Epoxidase activity (μg epoxide formed)	
	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
Before immunoadsorption	20	23	118	91	0.22	0.25
After immunoadsorption	≤3	<3	94	68	0.13	0.17
% Activity removed	>85	>87	20	25	39	32

Microsomes were prepared from Warfarin-treated rats (see section 2) and prothrombin precursor concentrations were determined both before and after immunoadsorption by activation with *Echis carinatus* venom [17], and the data expressed as thrombin units. Vitamin K-dependent protein carboxylation was assayed in 1.0 ml incubations containing solubilized microsomes (0.8 ml), NADH (1 mg), $NaH^{14}CO_3$ (50 μCi) and vitamin K_1 (50 μg) which were incubated at 27°C for 30 min. Incorporation of $H^{14}CO_3^-$ into protein was determined before and after immunoadsorption. Epoxidase activity was measured for 20 min at 37°C in 0.5 ml incubations containing 0.4 ml solubilized microsomes (=0.75 g liver/ml), 0.5 mg NADH, and 2 μg [^{3}H]vitamin K_1. Data are from 2 separate experiments of duplicate incubations differing by ≤10%

pool, the prothrombin precursors, results in a roughly equivalent decrease in both carboxylated protein and epoxidase activity, and suggests that the amount of epoxide found might be a direct function of the number of carboxylation events. The amount of carboxylation in this system can be directly varied by variation in the concentration of low molecular weight peptide substrates [15], and some stimulation of epoxidase activity above the high level observed in vitamin K-deficient animals has been observed by adding additional exogenous substrates [12,20]. The amount of carboxylation of endogenous substrates can be decreased to essentially zero by giving vitamin K-deficient animals an injection of the vitamin shortly before they are killed [20]. This allows the completion and release from the microsomes of accumulated precursors but leaves an induced carboxylase system. When carboxylation and epoxidation were measured in such a system (fig.1), it can be seen that increasing the substrate concentrations increased both carboxylation and epoxidation.

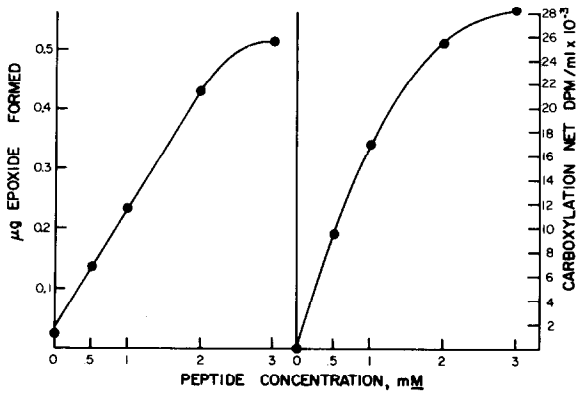

Fig.1. Effect of increasing substrate concentration on vitamin K-dependent carboxylation and vitamin K epoxidation. Vitamin K-deficient rats were given 1 mg vitamin K intracardially 30 min before they were killed and a liver microsomal preparation isolated and dissolved in Triton X-100. For the determination of epoxidase activity, incubations contained 0.4 ml microsomes solubilized in SIK–DTT buffer (see section 2) 0.5–3 mM Phe–Leu–Glu–Glu–Leu, 50 μg/ml Warfarin, 0.5 mM $NaHCO_3$ and 4 μg/ml [^{3}H]vitamin KH_2 in 0.53 ml total vol. Formation of vitamin K epoxide was measured after 30 min incubation at 27°C. Carboxylation of the added peptide substrate was measured under the same conditions with the substitution of 20 μCi $NaH^{14}CO_3$ for the unlabeled $NaHCO_3$ and unlabeled vitamin KH_2 for the [^{3}H]vitamin. The values plotted are means of duplicate assays which differ by <10%. A no vitamin K incubation blank of 2400 dpm/ml has been subtracted from all carboxylase values, and a 0 time blank of 0.15

4. Discussion

These data appear to offer conclusive proof that at least a portion of the epoxidase activity in rat liver microsomes is directly linked to the vitamin K-dependent carboxylation system. The removal of only a portion of the epoxidase activity by immunoadsorption with a specific prothrombin antibody is consistent with the observation that prothrombin precursors account for only ~25% of the vitamin K-dependent incorporation of $H^{14}CO_3^-$ into microsomal protein [17]. Precursors of other vitamin K-dependent proteins are present in microsomes of Warfarin-treated rats, and these are also substrates for the carboxylation reaction. The epoxidase activity observed after the removal of prothrombin precursors is presumably associated with the carboxylation of these other precursor proteins. The low epoxidase activity observed in normal rats or vitamin K-deficient rats given vitamin K is consistent with the low quantities of all the endogenous substrates for the carboxylation reaction present in these preparations. It is possible that treatment with the immunoadsorbent could have removed some of the carboxylase/epoxidase activity from the microsomal preparation and therefore caused a decrease in activity. The data in fig.1 suggest that this was not the basis for the alteration. When the number of carboxylation events was increased by increasing the substrate concentration with no other perturbation of the system, the epoxidase activity was increased. If these reactions were not coupled in some manner, a basis for such stimulation is not apparent.

How the formation of the epoxide is related to the carboxylation mechanism is not clear. A number of mechanisms have been proposed [22–24], and they are mainly based on the indirect evidence [11] that a hydroperoxide of the vitamin is an intermediate in both reactions. Epoxidation can proceed without carboxylation and in most studies the number of epoxidation events is considerably greater than the number of carboxylation events. When a pre-determined dilution [24] of the added $H^{14}CO_3^-$ by endogenous HCO_3^- was applied to the data in fig.1, there were 1.5–1.8 mol epoxide formed for each mole of CO_2 fixed. The data reported here do not, at the present time, lead to a unique model for the molecular role of vitamin K in the carboxylation reaction. Any mechanism proposed must, however, be consistent with these observations.

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